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Laser-flash-photolysis studies of *p*-cresol methylhydroxylase

Electron-transfer properties of the flavin and haem components

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p-Cresol methylhydroxylase, a heterodimer consisting of one flavoprotein subunit and one cytochrome *c* subunit, may be resolved into its subunits, and the holoenzyme may then be fully reconstituted from the pure subunits. In the present study we have characterized the reduction kinetics of the intact enzyme and its subunits, by using exogenous 5-deazariboflavin semiquinone radical generated in the presence of EDTA by the laser-flash-photolysis technique. Under anaerobic conditions the 5-deazariboflavin semiquinone radical reacts rapidly with the native enzyme with a rate constant approaching that of a diffusion-controlled reaction ($k = 2.8 \times 10^9 M^{-1} \cdot s^{-1}$). Time-resolved difference spectra at pH 7.6 indicate that both flavin and haem are reduced initially by the deazariboflavin semiquinone radical, followed by an additional slower intramolecular electron transfer ($k = 220 s^{-1}$) from the endogenous neutral flavin semiquinone radical to the oxidized haem moiety of the native enzyme. During the steady-state photochemical titration of the native enzyme at pH 7.6 with deazariboflavin semiquinone radical generated by light-irradiation the haem appeared to be reduced before the protein-bound flavin and was followed by the formation of the protein-bound anionic flavin radical. This result suggests that the redox potential of the haem is higher than that of the flavin, and that deprotonation of the flavin neutral radical occurred during the photochemical titration. Reduction kinetics of the flavoprotein and cytochrome subunits were also investigated by laser-flash photolysis. The protein-bound flavin of the isolated flavin subunit was reduced rapidly by the deazariboflavin semiquinone radical ($k = 2.2 \times 10^9 M^{-1} \cdot s^{-1}$), as was the haem of the pure cytochrome *c* subunit ($k = 3.7 \times 10^9 M^{-1} \cdot s^{-1}$). Flash-induced difference spectra obtained for the flavoprotein and cytochrome subunits at pH 7.6 were consistent with the formation of neutral flavin semiquinone radical and reduced haem, respectively. Investigation of the kinetic properties of the neutral flavin semiquinone radical of the flavoprotein subunit at pH 7.6 and at longer times (up to 5 s) were consistent with a slow first-order deprotonation reaction ($k = 1 s^{-1}$) of the neutral radical to its anionic form.

p-Cresol methylhydroxylase is an anaerobic dehydrogenase that catalyses the dehydrogenation and hydration of *p*-cresol to *p*-hydroxybenzaldehyde (Hopper, 1976, 1978). The enzyme consists of two unequal subunits of apparently the same M_r, a flavoprotein and a *c*-type cytochrome (Keat & Hopper, 1978). We have succeeded in resolving the enzyme into its subunits by a mild procedure, and upon mixing the component subunits in an appropriate medium full reconstitution of the enzymic activity and of other functional properties has been obtained (McIntire & Singer, 1982; Koerber *et al.*, 1985). This is the first instance of the successful reversible resolution of a flavocytochrome, and it has permitted comparison of the

Abbreviation used: deazaflavin, 5-deazariboflavin.

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catalytic properties of the pure flavoprotein subunit with those of the intact flavocytochrome. It was found in steady-state kinetic experiments that the maximal velocity of *p*-cresol oxidation by the flavoprotein subunit is only approx. 2% of that observed with the flavocytochrome and that the two differ in kinetic mechanism (McIntire & Singer, 1982; McIntire, 1983). Further, in stopped-flow experiments the rate of reduction of the flavoprotein subunit by the substrate was a small fraction of that observed with the intact flavocytochrome. These observations suggest that the cytochrome subunit modulates the catalytic properties of the flavoprotein.

It was desirable to extend these studies to a shorter time scale than those permitted by stopped-flow instrumentation, in order to measure the rate constants for reduction of the haem and the flavin components by an exogenous reductant and to determine if intramolecular electron transfer occurred on a short time scale. We were also interested in a further exploration of the regulatory influence of the cytochrome on the reactivity of the flavoprotein, as well as the possibility of the modulation of the reactivity of the cytochrome subunit by the flavoprotein. Laser-flash photolysis seemed the method of choice for such studies, since it has been successfully used for investigating the reduction kinetics of two other flavocytochromes, and, although electron transfer from one component to the other was not directly observed, a lower limit for the rate of intramolecular electron transfer from flavin to haem could be estimated (Cusanovich & Tollin, 1980; Tollin *et al.*, 1982; M. A. Cusanovich, T. E. Meyer & G. Tollin, unpublished work). The reducing agent used in the present study was 5-deazariboflavin semiquinone, an efficient and powerful reductant of flavoenzymes, which is produced on irradiation of deazaflavin in the presence of EDTA (Massey & Hemmerich, 1978). As is described below, rate constants for reduction of both the haem and the flavin components of *p*-cresol methylhydroxylase and its isolated flavoprotein and cytochrome subunits were measured, and the value of the rate constant for intramolecular electron transfer was directly determined. This is the first example in which electron transfer from the protein-bound flavin semiquinone to the haem has been directly observed and its rate constant determined for a flavocytochrome with a *c*-type haem.

Experimental

Materials

p-Cresol methylhydroxylase from *Pseudomonas putida* N.C.I.B. 9869, grown on 3,5-dimethylphenol, was isolated by a modification (Koerber *et al.*, 1985) of the procedure of Keat & Hopper (1978). The purity and specific activity of the enzyme were approximately the same as those of preparations isolated by the earlier method. The enzyme was resolved into its subunits by isoelectric focusing as described previously (McIntire & Singer, 1982). Glucose oxidase was from Miles Laboratories, crystallized bovine liver catalase (a 23mg/ml suspension) was from Sigma Chemical Co., and 5-deazariboflavin was a gift from (the late) Professor Peter Hemmerich (University of Konstanz, Konstanz, Germany).

Steady-state titrations

Freeze-dried enzyme (approx. 1mg) was dissolved in 100μl of 50mM-sodium phosphate buffer, pH7.6 and 15μl of 0.46mM-$K_3Fe(CN)_6$ was added. The solution was incubated for 2h at 0°C to ensure complete oxidation of the cytochrome component of the enzyme. Ferricyanide and ferrocyanide were removed by centrifugation through a 0.9cm × 5.5cm column of Sephadex G-50, equilibrated with the same buffer as above (Penefsky, 1977). A sample of the enzyme was diluted in an anaerobic cuvette to 1ml, so as to give a 4.4μM concentration of *p*-cresol methylhydroxylase. The solution contained 50mM-sodium phosphate buffer, pH7.6, 0.26μM-deazaflavin, 20mM-EDTA, 10mM-glucose, 20 units of glucose oxidase and 23μg of catalase. The last three components were added to ensure anaerobic conditions.

The anaerobic cuvette was evacuated and filled with argon six times. The volume of the solution thereby decreased, resulting in a final enzyme concentration of 5.25μM. The solution was then irradiated for various times at room temperature with two 15W Dayglow fluorescent bulbs, held at a distance of 5cm. This produced a constant steady-state concentration of the deazaflavin radical. The absorption spectrum was recorded after each irradiation.

Laser-flash photolysis

All flash experiments were performed at room temperature. The buffer used was 25mM-sodium phosphate, pH7.6, containing 20mM-EDTA and 100μM-deazaflavin. A few experiments were also performed with lumiflavin (50μM). Deoxygenation was achieved by bubbling argon through the flavin solution (typically 2ml) for about 45min. Concentrated protein solutions were added with the aid of a Hamilton syringe, and further degassing achieved by blowing argon over the surface of the solution. The formation and decay of the deazaflavin radical monitored at 500nm (or lumiflavin radical monitored at 560nm) on flash photolysis of the flavin solution was a good indicator of the degree of O_2 removal from the sample. Because of

the large slit width needed for data collection, the $\Delta\varepsilon_{550}$ value for the reduced haem is corrected from the true value of $19.4\,mM^{-1}\cdot cm^{-1}$ (McIntire, 1983) to an apparent value of $9.7\,mM^{-1}\cdot cm^{-1}$. Since the protein-bound neutral flavin radical has a relatively broad absorption spectrum, its absorption coefficient ($\Delta\varepsilon_{550} = 5.0\,mM^{-1}\cdot cm^{-1}$; McIntire, 1983) remains unchanged. A description of the laser-flash apparatus and the method of data collection and analysis has been given elsewhere (Bhattacharyya *et al.*, 1983).

Results

Laser-flash photolysis of the native enzyme

The initial experiments were aimed at determining which of the redox centres in the enzyme was more rapidly reduced by the deazaflavin radical generated by the laser flash (Edmondson *et al.*, 1972; Massey & Hemmerich, 1978) and whether intramolecular electron transfer between endogenous flavin and haem occurred under these conditions. The formation and decay of the deazaflavin radical was monitored at 500nm, which is an isosbestic point between the oxidized and reduced enzyme. At this wavelength, in the presence of oxidized *p*-cresol methylhydroxylase, an initial rapid increase in absorbance occurred, followed by an approximately first-order decay to the baseline (Fig. 1*a*). These changes corresponded to the formation and decay of the deazaflavin radical. The decay of the deazaflavin radical transient was much faster than the (second-order) decay of this species obtained in the absence of protein (Fig. 1*b*), indicating that the radical was being oxidized by the protein. Furthermore, the rate of decay of the deazaflavin radical was dependent on the concentration of oxidized protein. From a plot of the dependence of $k_{obs.}$ on the *p*-cresol methylhydroxylase concentration (inset of Fig. 1*a*), a second-order

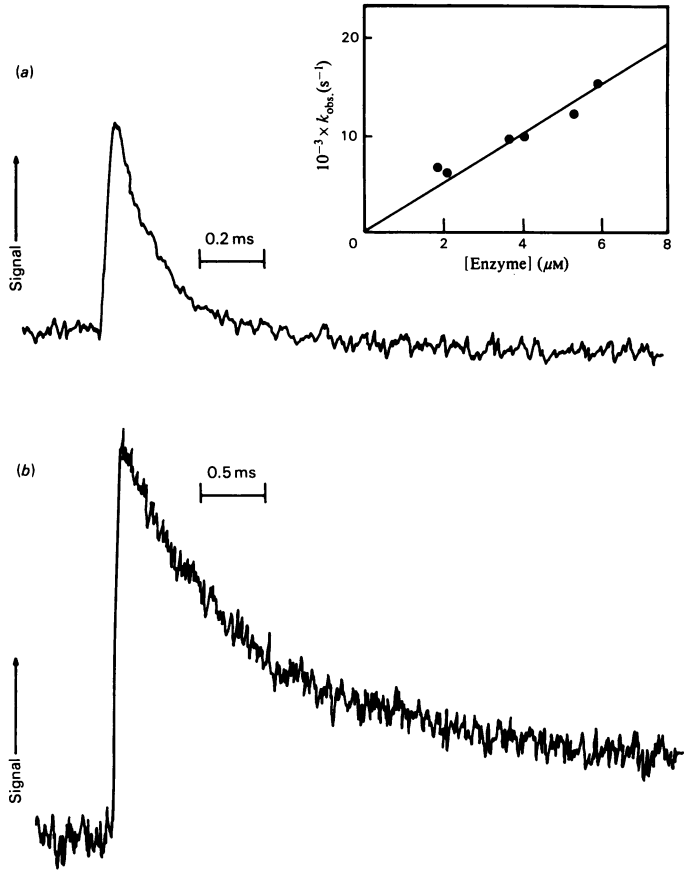

Fig. 1. *Flash-induced transient absorbance changes corresponding to the formation and decay of deazaflavin radical in (a) the presence and (b) the absence of oxidized p-cresol methylhydroxylase at pH 7.6*

The protein concentration in (*a*) was 2 μM oxidized *p*-cresol methylhydroxylase. 5-Deazaflavin concentrations and buffer conditions were as described in the Experimental section.

rate constant of $2.8(\pm 0.5) \times 10^9 M^{-1} \cdot s^{-1}$ was calculated for electron transfer from deazaflavin radical to the native enzyme.

In order to determine the initial product(s) of flavocytochrome reduction and whether any other electron-transfer processes occurred subsequently, the transient kinetics were examined at various wavelengths and at longer times (25ms). At pH7.6, and at 590nm (Fig. 2*a*), an initial rapid increase in absorbance was observed, followed by a slow exponential decay that eventually went below the pre-flash baseline. Such changes are consistent with the formation and re-oxidation of the neutral endogenous flavin semiquinone, resulting in the production of reduced haem. At 550nm, a wavelength at which haem reduction can be monitored, an initial rapid increase in absorbance was observed, followed by a slower exponential absorbance increase (Fig. 2*b*). The observed first-order rate constant for the slow rise at 550nm ($220 \pm 10 s^{-1}$), was the same (within experimental uncertainty) as the rate of re-oxidation of the protein-bound neutral flavosemiquinone monitored at 590nm (inset of Fig. 2*a*).

Fig. 3 shows flash-induced difference spectra obtained at pH7.6 for native *p*-cresol methylhydroxylase at $t = 0$ and 25ms after the laser flash. It is evident from the Figure that, subsequent to the decay of the deazaflavin radical, both haem and flavin reduction had occurred (●). From the relative absorption coefficients of reduced haem and neutral flavin semiquinone, a ratio of reduced haem to flavin radical of 2:1 was calculated (see the Experimental section). At longer times the difference spectrum resembled that of a typical oxidized minus reduced haem moiety (○), indicating that the flavin radical had transferred its electron to the oxidized haem. This is consistent with the slow first-order processes observed at 550 and 590nm. In order to determine whether this was

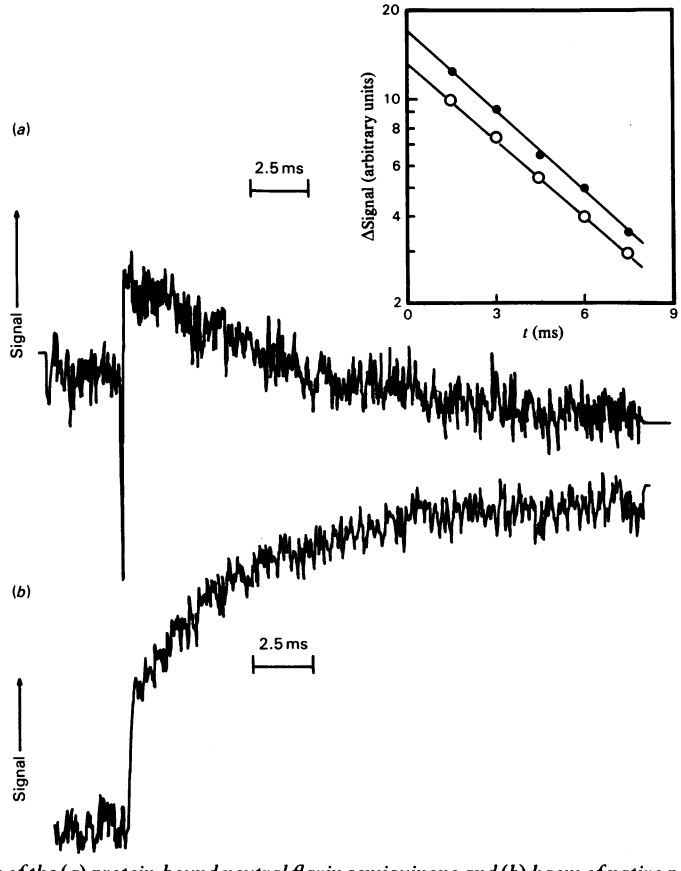

Fig. 2. *Transient kinetics of the (a) protein-bound neutral flavin semiquinone and (b) haem of native p-cresol methylhydroxylase at pH7.6*

Semiquinone kinetics were monitored at 590nm; haem reduction was monitored at 550nm. Protein concentrations in (*a*) and (*b*) were 20 μM. Buffer conditions were as described in the Experimental section. The inset shows semilogarithmic plots of the decay curves in (*a*) and (*b*).

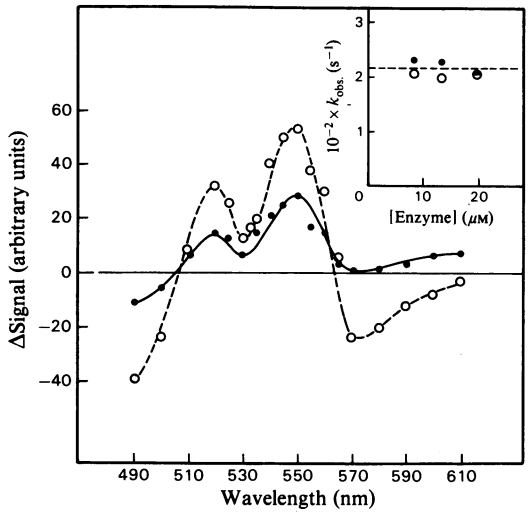

Fig. 3. *Flash-induced difference spectra (reduced minus oxidized) for native p-cresol methylhydroxylase obtained at t = 0 and t = 25 ms after the laser flash at pH 7.6*
The kinetic transients were extrapolated to zero time (●) to obtain the maximum amount of reduced species formed initially by the flash. ○ represents the reduced species present 25 ms after the laser flash. The protein concentration was 20 μM. Buffer conditions were as described in the Experimental section. The inset represents a plot of $k_{obs.}$ versus protein concentration, corresponding to the slow re-oxidation of protein-bound neutral flavin semiquinone (●) and the slow formation of reduced haem (○).

an intramolecular electron transfer the concentration of protein was varied. The rationale behind this experiment is that, if the formation of the observed spectral transient reflects a true first-order process, then the observed rate constant should be invariant with protein concentration. The inset shows that this was indeed the case, inasmuch that, when the protein concentration was varied over a 3-fold range, the rate constant for re-oxidation of the endogenous flavin semiquinone, as well as the slow phase of haem reduction, remained unchanged within experimental error. We thus conclude that this first-order rate constant corresponds to intramolecular electron transfer from the protein-bound neutral flavin semiquinone to the oxidized haem.

On progressive reduction of the haem component the magnitude of the decay of the protein-bound neutral flavin radical was considerably diminished, and when the haem was almost completely reduced (approx. 80%) no decay of the protein-bound flavin radical could be observed on a 25 ms time scale. This is consistent with the interpretation above.

The electron-transfer reaction of lumiflavin radical with oxidized *p*-cresol methylhydroxylase was also investigated by the laser-flash-photolysis technique. At pH 7.6, this reaction was second-order and occurred with a rate constant of $8.3(\pm 1.0) \times 10^7 M^{-1} \cdot s^{-1}$ (results not shown). The flash-induced difference spectrum obtained at pH 7.6 was qualitatively similar to that observed with deazaflavin, in that both endogenous flavin and haem reduction were found to occur (results not shown). The intramolecular electron transfer from protein-bound flavin semiquinone to oxidized haem could not be observed because of the reduction of the flavocytochrome by fully reduced lumiflavin, formed as a result of the rapid disproportionation reaction of the lumiflavin radical.

Photochemical titration of the flavocytochrome

Preliminary experiments indicated that both the flavin and the haem components of the unresolved enzyme could be photoreduced in the presence of deazaflavin and EDTA under steady-state conditions (McIntire, 1983). These studies have now been extended in order to determine the relative extent of reduction of these components under the conditions specified in the Experimental section. The reduction of the haem, monitored by increased absorbance at 552 nm, was complete in about 10 min, whereas complete reduction of the flavin, followed by bleaching at 450 nm, required about 35 min (Fig. 4). Fig. 4 also shows that the absorbance changes at 385 nm are triphasic. There is an initial decrease, coincident with the increase in absorbance at 552 nm, which is attributed to haem reduction. In the second phase an increase in absorbance is evident, and this can only be the result of the formation of the anionic flavin radical (McIntire *et al.*, 1981, 1985). In the final phase a decrease in absorbance occurs, indicating full reduction of the enzyme-bound flavin. The difference spectra of the enzyme after the first, second and third phases are shown in Fig. 5. Although the appearance of reduced flavin occurred later in the titration than that of the haem (cf. the changes at 552 and 450 nm in Fig. 4), the resulting time intervals for the three phases observed at 385 nm are about equal, indicating either that the rates of reaction of each enzyme species with the deazaflavin radical may be similar or that the reductions are limited by the rate of formation of the deazaflavin radical. A more detailed analysis of the relative rates was prevented by the fact that a number of redox reactions seem to occur concurrently: reactions of the deazaflavin radical with the flavoquinone, the semiquinone and the haem component, as well as rapid intermolecular electron transfer. These are discussed in more detail in the companion paper (McIntire *et al.*, 1985). The

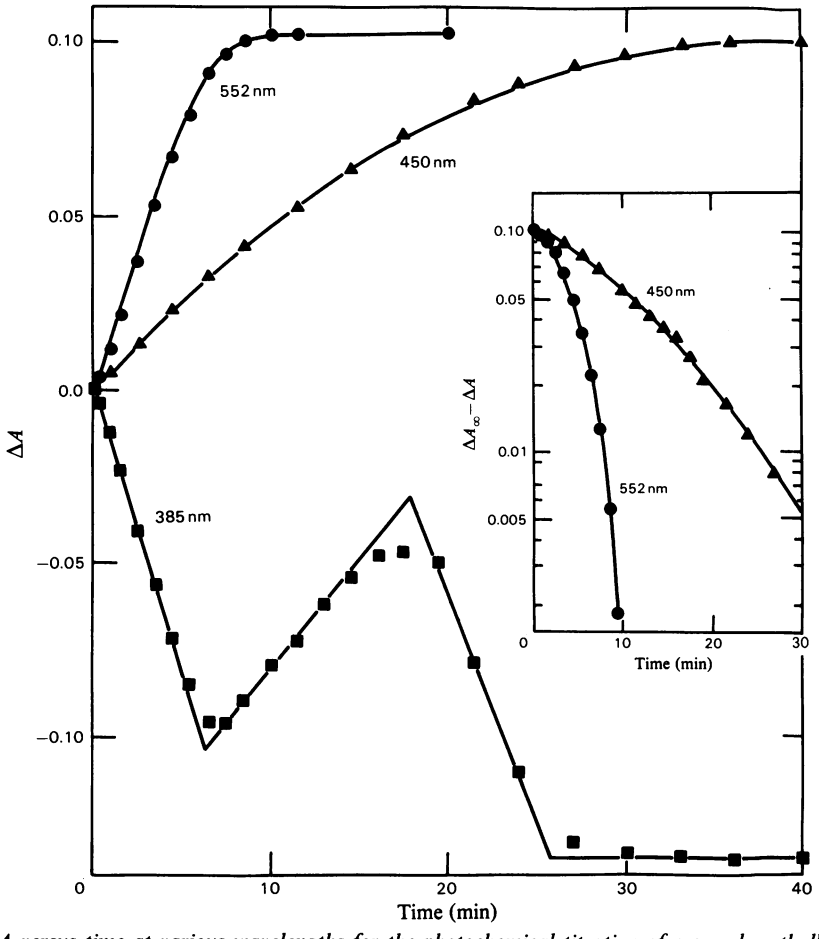

Fig. 4. *ΔA versus time at various wavelengths for the photochemical titration of p-cresol methylhydroxylase*
The absorbance values were obtained from difference spectra (reduced minus oxidized) at 552nm (●), 450nm (▲) and 385nm (■). Although the absorbance at 450nm decreased, for clarity the ΔA values are presented as positive numbers. Inset: semi-logarithmic plots of $\Delta A_\infty - \Delta A$ versus time for the data at 552nm and 450nm. The conditions of the experiment are described in the Experimental section.

occurrence of these multiple reactions seems to account for the characteristics of the absorbance changes described in Fig. 4.

The results of the steady-state experiment are consistent with the laser-flash-photolysis experiments, in which the reduced flavin is shown to transfer its electron to haem on a millisecond time scale. Thus, in a steady-state experiment, the haem was reduced before the flavin, and deprotonation of the protein-bound neutral radical had occurred before the spectrum could be recorded. We can conclude from these experiments that the redox potential of the haem is higher than that of the flavin, and that there is communication between the flavin and haem moieties of the native enzyme.

Flash-photolysis studies of the cytochrome subunit

As was the case with the flavocytochrome, the reaction of deazaflavin radical with the oxidized cytochrome subunit was monitored at 500nm. Kinetic traces showed an initial increase in absorbance, followed by an approximately first-order decay to the baseline, again consistent with rapid formation and re-oxidation of the deazaflavin radical by the oxidized cytochrome subunit. The rate of oxidation of the deazaflavin radical increased with increasing concentration of the cytochrome subunit, and a plot of $k_{obs.}$ versus oxidized protein concentration yielded a second-order rate constant of $3.7(\pm 0.4) \times 10^9 M^{-1} \cdot s^{-1}$ (inset of Fig. 6). This rate constant is approximately the same as that observed with the intact flavocytochrome.

The course of reaction between deazaflavin radical and the cytochrome subunit was monitored at several wavelengths, and a flash-induced differ-

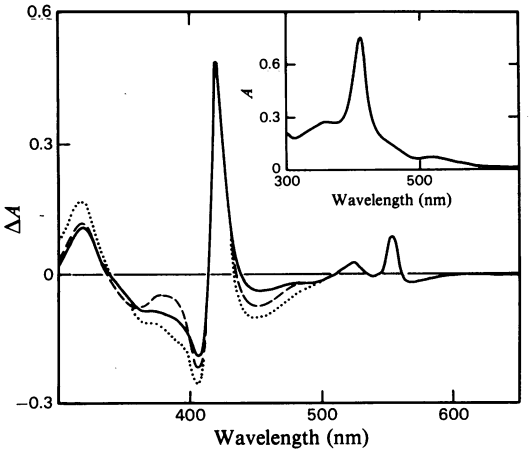

Fig. 5. *Difference spectra (reduced minus oxidized) of p-cresol methylhydroxylase after the first, second and third phases for the photochemical titration*

The spectra are for the absorbance changes recorded after 8.5min (——), 17.5min (– – – –) and 40min (·····). Note that in the 385nm region the absorbance first decreases, then increases, and finally decreases again. The inset shows the absorption spectrum of the oxidized flavocytochrome.

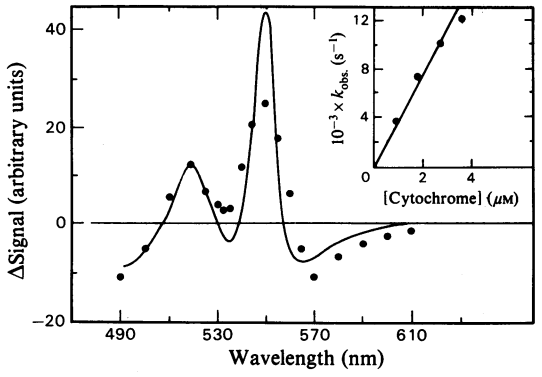

Fig. 6. *Flash-induced (reduced minus oxidized) difference spectrum obtained for the cytochrome subunit of p-cresol methylhydroxylase at pH7.6*

The ● symbols represent the data for the flash experiment. The cytochrome subunit concentration was 13 μM. The continuous line corresponds to an oxidized-minus-dithionite-reduced cytochrome subunit difference spectrum obtained in a Cary 14 spectrophotometer. The inset shows a second-order plot for the reaction between deazaflavin radical and oxidized cytochrome subunit.

ence spectrum was obtained at pH7.6 (Fig. 6). Also shown for comparison is a dithionite-reduced-minus-oxidized difference spectrum obtained in a Cary 14 spectrophotometer. Considering the poorer spectral resolution of the laser-photolysis apparatus, the agreement is satisfactory.

Laser-flash-photolysis studies of the flavoprotein subunit

Kinetic traces obtained from laser-flash experiments performed at 500nm were consistent with the re-oxidation of the deazaflavin radical by the oxidized flavoprotein subunit. The rate of decay of deazaflavin radical increased with increasing concentration of oxidized flavin subunit, and a plot of $k_{obs.}$ versus protein concentration yielded a second-order rate constant of $2.2(\pm 0.1) \times 10^9 M^{-1} \cdot s^{-1}$ (inset of Fig. 7) for the electron-transfer reaction. This is very similar to that obtained with the holoprotein.

Fig. 7 shows the flash-induced difference spectrum obtained for the flavoprotein subunit at pH7.6 measured 5ms after the laser flash. The decrease in absorbance below 495nm is consistent with reduction of the protein-bound flavin moiety. The spectral changes between 495 and 610nm are consistent with the formation of protein-bound neutral flavin radical (Müller *et al.*, 1972).

The flash experiments described above indicated that the neutral flavin radical was stable up to 50ms after the laser flash (results not shown). On the other hand, steady-state phototitration experiments have shown that only the anionic form of the flavin radical can be observed upon irradiation. A possible explanation for this discrepancy is that the neutral radical, which is initially produced by hydrogen transfer from the deazaflavin radical, is slowly deprotonated to the anionic form. This prompted us to investigate the kinetics of the laser-produced flavin neutral radical at much longer

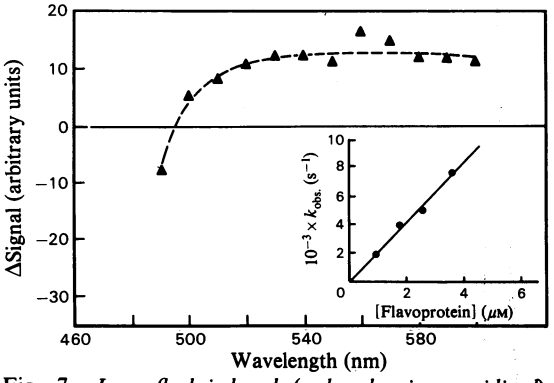

Fig. 7. *Laser-flash-induced (reduced minus oxidized) difference spectrum for the flavoprotein subunit of p-cresol methylhydroxylase*

The spectrum was obtained by plotting the signal obtained 5ms after the laser flash as a function of wavelength. The protein concentration was 20 μM. Buffer conditions were as described in the Experimental section. The inset shows a second-order plot corresponding to the reaction between deazaflavin radical and oxidized flavoprotein subunit.

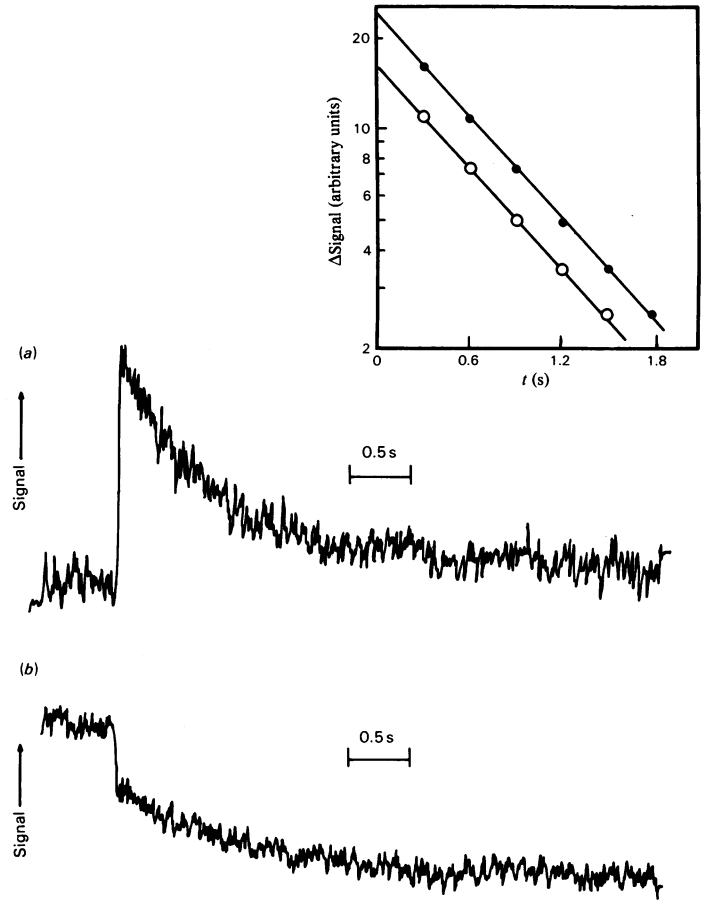

Fig. 8. *Absorbance changes corresponding to the formation and decay of the protein-bound neutral flavin semiquinone of the flavoprotein subunit of p-cresol methylhydroxylase monitored at (a) 590nm and (b) 480nm*
The protein concentration was 20 μM. Buffer conditions were as in Fig. 7. The inset corresponds to semi-logarithmic plots of the slow transient kinetics observed at 590nm (●) and at 480nm (○).

times. Any deprotonation of this species would be reflected in a loss of absorbance at 590nm. Furthermore, the decay should be a first-order process. Fig. 8 shows the kinetic behaviour of the flavin radical on a 5s time scale. An initial rapid increase in absorbance occurred, followed by an almost complete first-order decay ($k = 1\,s^{-1}$) to the baseline (inset of Fig. 8). This result is consistent with the rapid formation of the neutral radical and a slow deprotonation to the anionic form. That the observed decay in absorbance at 590nm is not due to disproportionation, or to re-oxidation of the flavin radical by some external oxidant, is shown by the kinetics at 480nm, a wavelength at which oxidized flavin absorbs. On a 5s time scale a rapid decrease in absorbance was observed at this wavelength, followed by a much slower additional bleaching (Fig. 8). The rate constant for the slow bleaching was the same within experimental error as the rate constant for the slow decay observed at 590nm (inset of Fig. 8). If the slow decay at 590nm resulted in the formation of oxidized flavin, then, at 480nm on a 5s time scale, an increase in absorbance, rather than decolorization, should have been observed. We thus attribute the observed first-order process to the slow deprotonation of the neutral flavin radical to its anionic form. This interpretation is consistent with the results of the steady-state photochemical titration experiments.

Discussion

The results presented above demonstrate the utility of the laser-flash-photolysis technique in measuring both the inter- and intra-molecular processes involved in the electron-transfer reactions of native *p*-cresol methylhydroxylase and its individual subunits. The rate of reduction of the native enzyme by deazaflavin semiquinone was

close to the diffusion limit, and the difference spectra indicated that both flavin and haem were reduced simultaneously. The simplest explanation of this latter fact is that both redox centres are exposed at the enzyme surface and directly interact with the deazaflavin radical. Since twice as much reduction of haem occurs relative to flavin, this would imply a greater reactivity of the haem moiety with the deazaflavin radical. However, it should be kept in mind that we can only measure the formation of the neutral flavin semiquinone, and thus, if some of the anionic form were also being produced, we may have underestimated the extent of flavin reduction.

We were also able to observe intramolecular electron transfer from the protein-bound flavin semiquinone to the oxidized haem moiety in the native enzyme. Although the redox potential of the flavin has not been measured, the direction and extent of the observed intramolecular electron-transfer reaction indicates that the one-electron potential of the protein-bound flavin is appreciably lower than that of the haem ($E_{m,7} = +250$mV). This result is consistent with the results obtained from the steady-state experiments.

The reduction kinetics of native *p*-cresol methylhydroxylase may be compared with those of two other flavocytochromes, isolated from *Chromatium vinosum* and *Chlorobium thiosulfatophilum* (Cusanovich & Tollin, 1980; Tollin *et al.*, 1982; M. A. Cusanovich, T. E. Meyer & G. Tollin, unpublished work). The reaction of all three flavocytochromes with deazaflavin radical is second-order, with rate constants ranging from $3 \times 10^8 M^{-1} \cdot s^{-1}$ (*Chromatium*) to $2.8 \times 10^9 M^{-1} \cdot s^{-1}$ (*p*-cresol methylhydroxylase). *p*-Cresol methylhydroxylase is also more reactive with lumiflavin semiquinone than are the other flavocytochromes (M. A. Cusanovich, T. E. Meyer & G. Tollin, unpublished work). Furthermore, the initial electron-transfer reaction from deazaflavin radical to the oxidized protein, in all three flavocytochromes, leads to the formation of both protein-bound neutral flavin semiquinone and reduced haem. In contrast with the other two flavocytochromes, we are able to observe directly the intramolecular electron transfer from flavin radical to oxidized haem in *p*-cresol methylhydroxylase. At least with the *Chromatium* flavocytochrome, an analogous intramolecular reaction cannot be observed (M. A. Cusanovich, T. E. Meyer & G. Tollin, unpublished work). Since the second-order rate constants for reduction of the cytochrome and flavin subunits are approximately the same as that observed for the native enzyme, little or no change of the reactivity of the haem or flavin towards deazaflavin semiquinone occurred upon subunit association. By using laser photolysis we were able to observe the deprotonation of the neutral semiquinone of the flavoprotein subunit to its anionic form, occurring with a rate constant of $1 s^{-1}$. In the case of the holoprotein, this reaction cannot be directly observed because of the intramolecular electron transfer to the haem. However, when the haem is fully reduced, the neutral flavin radical formed in the flash experiment was stable for 25ms. Since only the anionic flavin radical was observed in the photochemical titration, a fairly rapid deprotonation (on the time scale of minutes) must also occur in the case of the flavocytochrome. The formation of the anionic flavin semiquinone was also seen during titrations of the flavocytochrome with *p*-cresol (McIntire *et al.*, 1984). Stopped-flow studies and other experiments (McIntire, 1983; McIntire *et al.*, 1984) have shown that with most substrates only the anionic flavin semiquinone can be detected with *p*-cresol methylhydroxylase, although with *p*-cresol the formation of another transient has been shown to precede this event. However, the spectral properties of this transient were not consistent with those of the neutral protein-bound radical.

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